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Title: Fermentation and alternative oxidase contribute to the action of amino acid biosynthesis inhibiting herbicides

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Fermentation and alternative oxidase contribute to the action of amino acid biosynthesis-inhibiting herbicides

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SUMMARY

Acetolactate synthase inhibitors (ALS-inhibitors) and glyphosate are two classes of herbicide that act by the specific inhibition of an enzyme in the biosynthetic pathway of branched-chain or aromatic amino acids, respectively. The physiological effects that are detected after application of these two classes of herbicides are not fully understood in relation to the primary biochemical target inhibition, although they have been well documented. Interestingly, the two herbicides' toxicity includes some common physiological effects suggesting that they kill the treated plants by a similar pattern despite targeting different enzymes. The induction of aerobic ethanol fermentation and alternative oxidase are two examples of these common effects. The objective of this work was to gain further insight into the role of fermentation and alternative oxidase induction in the toxic consequences of ALS-inhibitors and glyphosate. For this, Arabidopsis T-DNA knock-out mutants of alcohol dehydrogenase 1 and alternative oxidase 1a were used. The results found in wild-type indicate that both glyphosate and ALS-inhibitors reduce ATP production by inducing fermentation and alternative respiration. The main physiological effects in the process of herbicide activity upon treated plants were accumulation of carbohydrates and total free amino acids. The effects of the herbicides on these parameters were less pronounced in mutants compared to wild-type plants. The role of fermentation and alternative oxidase regarding pyruvate availability is also discussed.

Keywords: Glyphosate, acetolactate synthase inhibitors, ethanol fermentation, *Arabidopsis thaliana*, physiological effects

Abbreviations GABA, γ–aminobutyric acid; GLP, glyphosate; IMX, imazamox; ADH, alcohol dehydrogenase; ALS, acetolactate synthase; AOX, alternative oxidase; EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; PDC, Pyruvate decarboxylase

1. Introduction

Herbicides that inhibit amino acid biosynthesis are useful tools in weed management and have been particularly successful as their biochemical target can only be synthesized by plants and microorganisms, thus reducing the probability of a toxic effect on mammals. These herbicides were developed in the early 1970s and are still of great agronomic and commercial importance. There are several types of herbicides whose target or primary site of action is the specific inhibition of enzymatic activity in biosynthetic pathways of amino acids. In this context, two important sites of herbicide action are acetolactate synthase (ALS, EC 4.1.3.18; also referred to as acetohydroxyacid synthase) and 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; EC 2.5.1.19), key enzymes in branched-chain and aromatic amino acid biosynthetic pathways, respectively (Duke 1990).

ALS is the first enzyme in the biosynthesis of the three branched-chain amino acids valine, leucine and isoleucine. ALS-inhibitors include several classes of chemicals and have become one of the most widely used types of herbicides due to their wide-spectrum weed control activity, high crop selectivity, low application doses and low toxicity to mammals (Zhou et al. 2007). More than 50 commercial herbicide ingredients have ALS as their primary target. EPSPS is a key enzyme in the biosynthesis of aromatic amino acids (tyrosine, phenylalanine and tryptophan) and is inhibited by the herbicide glyphosate (GLP) (Steinrucken and Amrhein 1980). GLP (*N*-(phosphonomethyl)glycine) is a wide-spectrum, non-selective post-emergence herbicide and is currently the most commonly used herbicide in the world, particularly since the introduction of transgenic GLP-resistant crops (Powles 2008).

The herbicide action cannot be considered only in terms of interaction at a target site. The interaction can be viewed as the first step that is followed by a series of physiological consequences that result in death of the plant. A major limitation to understanding herbicide action is the lack of detailed knowledge of such consequences. Some possible causes of plant death as a consequence of the inhibition of biosynthetic pathways are the accumulation of toxic precursors or intermediates, end-product depletion, diversion of precursor into other products or deregulation of that pathway.

Although the specific biochemical targets of herbicides inhibiting branched or aromatic amino acid biosynthesis are well documented, the ultimate cause of plant death is not known. For both types of amino acid biosynthesis -inhibiting herbicides, the sequence of events that leads from herbicide application to plant death is still unclear. To understand the physiological effects that are involved in the lethal process after the herbicide treatment is important, as it can lead to their more rational use, and also because it can help in the development of new compounds with similar herbicidal activities but with different enzyme inhibition targets to avoid weed resistance. Several common physiological effects produced by ALS and EPSPS-inhibitors have been described in the literature, leading to the conclusion that their toxicities share certain characteristics. The common effects include growth arrest followed by the slow death of treated plants (Gruys and Sikorski 1999; Wittenbach and Abell 1999), a general increase in total free amino acid content (Shaner and Reider 1986; Wang 2001; Zulet et al. 2013a) and the accumulation of some secondary metabolites, such as quinate, a compound synthesized in a lateral branch of the shikimate pathway (Orcaray et al. 2010).

Moreover, ALS-inhibitors and GLP have been reported to impair carbon metabolism. They cause

growth arrest in roots leading to an accumulation of unused carbohydrates, which in turn triggers a decrease in sink strength with a consequent accumulation of carbohydrates in the leaves (Zabalza et al. 2004; Orcaray et al. 2012). Another common effect on the roots of plants treated with ALS and EPSPS-inhibitors is the induction of fermentation and the alternative respiratory pathway; both of which are low-ATP producing pathways (Gaston et al. 2002, 2003; Zabalza et al. 2005; Orcaray et al. 2012), These metabolic impairments indicate that the effect of these herbicides on primary plant metabolism has broader physiological consequences than a lack of certain amino acids alone.

What induces the fermentation and alternative respiration in roots following ALS or EPSPS inhibition remains unknown. The induction of fermentation under aerobic conditions after ALS inhibition has been related to pyruvate availability, as pyruvate is a common substrate of both ALS and PDC (pyruvate decarboxylase, the first enzyme in ethanol fermentation, EC 4.1.1.1). Therefore ALS inhibition would mean an increase in the availability of pyruvate to be used by other enzymes, such as PDC. Moreover, pyruvate has been reported to be an allosteric activator in the activity of alternative oxidase (AOX) (Millar et al. 1993) (Vanlerberghe et al. 1995). However, induced fermentation after treatment with EPSPS -inhibiting GLP is not expected to be related to increased pyruvate availability as this herbicide does not inhibit any pyruvate-consuming enzymes, so what triggers fermentation after the application of this herbicide is unknown.

It is important to elucidate the role of induction of these two pathways (fermentation and alternative respiration) after the application of the two types of herbicides. Two, non-contradictory explanations can be considered. Firstly, the induction of these two pathways could be a plant defense mechanism that promotes better tolerance of the herbicide, and/or secondly, it could be a consequence of the herbicidal activity, thus contributing to the chemical's toxicity.

To achieve insights into the common series of consequences of amino acid biosynthesis -inhibiting herbicides we focused on the induction of fermentation and alternative oxidase. We evaluated whether the effect of the herbicides was increased or decreased by reduced fermentation activity or reduced AOX expression in mutant plants. For this purpose, fermentation, alternative oxidase, carbohydrate and amino acid content were compared in Arabidopsis lines (wild-type, *adh* and *aox1a* knock outs) treated with imazamox (IMX, an ALS-inhibitor) (Ohba et al. 1997) or GLP. Here, we show how Arabidopsis mutant plants treated with these amino acid biosynthesis -inhibiting herbicides respond differently than wild-type plants. Some of these parameters were less affected in the mutants compared to the wild-type lines, providing evidence that fermentation and alternative oxidase contribute to herbicide action.

2. Materials and Methods

2.1 Plant material and treatment application

Arabidopsis thaliana ecotype Col-0, its alcohol dehydrogenase (*adh*, NASC N552699 (Banti et al. 2008)) and its alternative oxidase 1a (*aox1a*,, SALK_084897 (Strodtkötter et al. 2009)) knock out mutants were grown in aerated hydroponic culture. Growth conditions were 150 μmol m^{-2} s^{-1} PPF, 65 % RH and 25/20ºC day/night. The plants were maintained in a 12 h/12 h day/night photoperiod for the first 4 weeks and grown in 8/16 h day/night photoperiod afterwards to prevent flowering. The nutrient solution was slightly modified from (Loqué et al. 2003): 1 mM NH_4NO_3, 1 mM KH_2PO_4, 1 mM $MgSO_4$, 250 mM $CaCl_2$, 0.1 mM Na-Fe-EDTA, 50 mM KCl, 50 mM H_3BO_3, 5 mM $MnSO_4$, 1 mM $ZnSO_4$, 1 mM $CuSO_4$, and 0.1 mM $(NH_4)_6Mo_7O_{24}$. When plants were approx. 8 weeks old IMX or GLP were applied. The experiment was performed in duplicate. Throughout the course of the experiment the plants remained in the rosette vegetative phenological stage.

The two herbicides were applied to the nutrient solution as commercial formulations: 1.5 mg active ingredient L^{-1} (4.8 μM) IMX (Pulsar®40, BASF Española SA, Barcelona, Spain) and 50 mg active ingredient L^{-1} (220.8 μM) GLP (Roundup®Plus, MONSANTO Agricultura España SL, Madrid, Spain). Preliminary studies were conducted to find comparable doses of IMX and GLP.

Net carbon dioxide assimilation rates were measured from the youngest, fully-expanded leaf in intact plants using a portable ADC-LCpro+ system equipped with an Arabidopsis chamber (ADC BioScientific Ltd, Herts, UK). Measurements were made in the growth chamber under growing conditions (400 ppm CO_2, 25ºC leaf temperature, 1.1 kPa VPD).

Root samples were taken at day 3 after application of the treatment. Plant material was immediately frozen in liquid nitrogen and stored at -80 ºC. Some material was dried for 48 hours at 75 - 80 ºC in order to obtain the fresh weight/dry weight ratio.

2.2 ADH and PDC activities and Western immunoblot assay

PDC and ADH activities were assayed in desalted extract as described by (Gaston et al. 2002). Total protein was isolated from roots as described by (Zabalza et al. 2005). Western blots were performed according to standard techniques (Zabalza et al. 2009). PDC and ADH antibodies from Agrisera (Vännäs, Sweden) were used at dilutions of 1:10,000 and 1:3,000, respectively. Goat Anti-Rabbit IgG HRP Agrisera (Vännäs, Sweden) was used as the secondary antibody at a dilution of 1:20,000 and bands were visualized using ECL Prime Western Blotting Reagents (GE Healthcare, Buckinghamshire, UK) and a Bio-Rad ChemiDoc Imaging system (ChemiDoc, Biorad, USA).

2.3 Real-time qRT-PCR analysis

Total RNA was extracted using the NucleoSpin RNA Plant kit (Macherey-Nagel, GMBH) according to the manufacturer's instructions. 13 μg of RNA (DNA free) was reverse transcribed into cDNA using the

SuperScript II Reverse Transcriptase kit (Invitrogen, CA, USA). Real-time PCR amplification was carried out with the ABI PRISM 7900 sequence detection system (Applied Biosystems, Foster City, CA, USA) using a SYBR Green master mix (Takara, Shiga, Japan) and the primers described in Supplemental Table 1. PP2A (AT1G13320) selected from the list presented by Czechowski et al. (2005), was used as a housekeeping gene once it was checked that Ct values of PP2A were stable. Relative quantification of the expression of each individual gene was performed using the comparative threshold cycle method.

2.4 Carbohydrate determination

Soluble carbohydrate (glucose, fructose and sucrose) content was determined in ethanol-soluble extracts and the ethanol-insoluble residue was extracted for starch analysis (Zabalza et al. 2004). The determination of starch and soluble sugar concentration was performed using capillary electrophoresis as described by (Zabalza et al. 2004).

2.5 Amino acid and glutathione content determination

Amino acids were extracted in 1M HCl. After protein precipitation, amino acid concentrations were analyzed in the supernatant. After derivatization with FITC, amino acid content was measured using capillary electrophoresis equipped with a laser-induced fluorescence detector, as described elsewhere (Zulet et al. 2013b). Cysteine and glutathione content were determined from the same acid extracts derivatized with 5-iodoacetamide fluorescein and reduced with tributylphosphine as described by (Zinellu et al. 2005).

2.6 Statistical analysis

Each mean value was calculated using different samples from individual plants as replications. The results were subjected to a separate one-way ANOVA analysis (SPSS Statistics 22) and the means were separated using the Tukey test (Significance level (p value) of 0.05). In Figures 1, 3, 5 and Supplemental Figure 1 the symbol $\triangledown$ highlights the significant differences, if detected, between untreated mutant plants and untreated wild-type plants (i.e., control plants). For each of the three genotypes investigated, significant differences between the herbicide treated plants and their respective control plants are highlighted in the figures using a different symbol for each herbicide.

3. Results

3.1 Dose determination

Preliminary studies were conducted to determine the IMX and GLP concentrations that induce similar effects to those previously described in pea plants (Zabalza et al. 2004, 2005; Orcaray et al. 2012; Zulet et al. 2013a). We found that 4.8 μM IMX and 220.8 μM GLP have similar effects and that both treatments caused plant death at 20 days, when photosynthesis was almost zero in the three genotypes evaluated (Supplemental Fig. S1). Samples were taken after three days, before obvious visual plant death was observed. This time point was chosen in order to allow us to evaluate physiological and biochemical plant responses induced by the herbicide but not directly resulting from cell death.

3.2 Ethanol fermentation and hypoxia markers

The relative expression level, amount of protein and activity of enzymes related to fermentation were evaluated in aerated roots of wild-type (Col-0) and mutant (*adh* and *aox1a*) Arabidopsis 3 days after treatment with IMX or GLP (Fig. 1). The two enzymes of the ethanol fermentation pathway, pyruvate decarboxylase (PDC) and ADH, were evaluated by relative expression level of transcripts (Fig. 1A), quantity of protein and enzymatic activity (Fig. 1B). The expression level of two of the four genes belonging to the PDC gene family in Arabidopsis (Kursteiner et al. 2003) were analyzed. In wild-type roots, PDC and ADH activities increased after IMX treatment in concordance with the expression pattern of one of the genes of PDC (*PDC1)* and *ADH1* and quantity of protein. In wild-type plants, GLP treatment induced an increase in PDC activity while the ADH activity was not significantly different to that of untreated plants. In these plants, PDC and ADH protein quantity were increased after GLP treatment.

Comparing control plants of wild-type and mutant genotypes, the expression of *PDC1* and *PDC2* was similar but the levels of PDC protein and PDC activity were higher in mutant roots than in wild-type ones. As expected, *adh* mutant did not have transcripts of the *ADH1* gene

IMX induced the high relative expression level of *PDC1* in the wild-type; however this level of induction was not present in either of the mutants. In *PDC2* transcripts, an induction after GLP was observed in *adh* and *aox1a* mutants that was not detected in the wild-type. The increased activity and amount of PDC protein detected in the wild-type after IMX treatment was also observed in the mutants, whereas with GLP treatment only the wild-type showed increased activity and PDC content while *adh* and *aox1a* mutants had similar levels as the control plants. The increased activity and amount of ADH protein detected in the wild-type plants 3 days after treatment with IMX was also observed in the *aox1a* mutant.

Lactic fermentation was also evaluated by analyzing the expression of one gene of lactate dehydrogenase (*LDH1*) using qPCR (Fig. 1A). Induction of the expression of this gene due to IMX as observed in the wild-type was less pronounced in *adh* mutant and no induced expression was observed in *aox1a* mutant; GLP treated plants presented the same effects on *LDH1* gene expression independently of the genotype.

Another quite important pathway related to fermentation is the production of alanine from pyruvate through alanine aminotransferase (AlaAT). Four genes code for the four isoenzymes that form the AlaAT family in Arabidopsis (Igarashi et al. 2003; Liepman and Olsen 2003). The *AlaAT1* gene has been reported to be the most expressed isoenzyme in Arabidopsis roots (Miyashita et al. 2007). Nevertheless, in this work, no differences were found between the relative expression levels of the genes *AlaAT1* and *AlaAT2* (data not shown). No effects of the herbicides were observed in the *AlaAT1* relative expression level, therefore the *AlaAT2* gene was chosen to evaluate the pattern of alanine aminotransferase because it showed a response to the herbicide stress (Fig. 1A). The herbicide IMX produced a striking induction of the *AlaAT2* relative expression level in both wild-type plants and mutants.

In order to confirm that the plants experience low oxygen stress a hypoxia marker was used. The relative expression level of non-symbiotic hemoglobin (*HB1*) was quantified (Fig. 1A) as it has been reported that biosynthesis of this protein is induced under low oxygen conditions (Branco-Price et al. 2005; Loreti et al. 2005; van Dongen et al. 2009). The expression of *HB1* has also been used as marker to study the expression of anaerobic genes under low oxygen conditions (Licausi et al. 2010). Our results revealed an induction of *HB1* expression in the wild-type with both herbicides, suggesting an expression pattern similar to low oxygen even though the plants were well aerated throughout the experiment (oxygen at 100% air saturation). Nevertheless, the induced increase of *HB1* transcription observed in treated wild-type plants was not observed in mutants where the expression of the *HB1* gene was similar with or without herbicides.

3.3 AOX expression

In order to discover the effects of GLP and IMX herbicides on AOX, the relative expression levels of the five AOX genes of Arabidopsis (AOX1a, 1b, 1c, 1d and AOX2) (Clifton et al. 2006) were analyzed by qPCR in wild-type and mutant plants 3 days after being treated with IMX or GLP (Fig. 2). In wild-type roots *AOX1a* gene expression was the most abundant and we confirmed that *aox1a* did not have transcripts for this gene. The herbicide IMX produced a significant induction of the transcripts of *AOX1a, 1c, 1d* and *AOX2*. IMX treatment showed a stronger effect than GLP on the relative expression levels of *AOX* genes. GLP had no effect on *AOX1* expression and it even decreased the relative *AOX2* gene expression in comparison with the control. In general, the effect of GLP and IMX on mutants was the same as observed on the wild-type and in some cases less pronounced.

3.4 Carbohydrate content

As carbohydrate accumulation has previously been shown to be induced by the application of amino acid biosynthesis -inhibiting herbicides, both branched-chain (Zabalza et al. 2004) and aromatic (Orcaray et al. 2012) amino acids, it can be used as a physiological marker of herbicide toxicity.

Carbohydrate content (glucose, fructose, sucrose and starch) was measured in Arabidopsis wild-type and mutant roots 3 days after being treated with IMX or GLP (Fig. 3). Both herbicides produced an increase in carbohydrate content in wild-type plants, however, the effect was not the same for both herbicide treatments

as the content of two soluble carbohydrates (fructose and sucrose) increased with GLP and starch content only increased not significantly after IMX treatment. Comparison of the control plants (untreated) for the three genotypes revealed that carbohydrate levels were similar, although several differences were detected: control *aox1a* mutants presented significantly higher contents of fructose and glucose compared to wild-type plants. No accumulation of sucrose was observed when the herbicides were applied to mutant plants; unlike in the wild-type plants after GLP treatment, and glucose and fructose content were even lower after GLP application than in untreated plants for both *adh* and *aox1* mutants.

3.5 Amino acid content

The treatment with amino acid biosynthesis -inhibiting herbicides also results in amino acid accumulation, as described previously (Zabalza et al. 2005; Orcaray et al. 2012), and so this parameter can also be used as a physiological marker of herbicidal activity.

Free amino acid content was measured in plants 3 days after being treated with IMX or GLP (Fig. 4). Figure 4 shows the most representative results of amino acid content. Total free amino acid content is shown and as alanine has been related to fermentation, the absolute content of this amino acid is also included. As the impact of changes of specific amino acids could be masked by the general increase of the total free amino acid pool content, each specific group of amino acids has been expressed as a percentage of the total free amino acids instead of an absolute value. Amino acids whose biosynthesis is inhibited were grouped and shown as branched-chain (val, leu, ile) and aromatic (phe, tyr, trp). Moreover two other groups previously reported to be affected by the herbicides (Orcaray et al., 2010) are included: acidic (glu, asp), and amide (gln, asn).

The content of each individual amino acid is provided in Supplemental Figure 3. Amino acids are presented and grouped by the main biosynthetic pathways. A simplified overview of all the amino acid biosynthesis is shown in Supplemental Figure 2.

Almost all the parameters shown in Figure 4 were similar for the control plants representing the three genotypes, except for the group of the aromatic amino acids in the *aox1a* genotype. In wild-type plants, IMX produced an expected significant decrease of the relative percentage of branched-chain, aromatic and acidic amino acids, while the amides increased. Whereas, GLP produced, in the same genotype, a decrease in acidic amino acids and did not affect the ratio of the aromatic amino acids.

Branched-chain amino acid content was lower in all genotypes after IMX or GLP treatment. IMX treatment also produced lower relative percentages of aromatic amino acids in all genotypes compared to untreated control plants, whereas after GLP treatment a lower content was observed in the mutants but not in the wild-type plants. With respect to acidic amino acids; a lower content was detected in wild-type plants following application of both herbicides, while in mutants this reduced content (compared to the controls) was only observed in IMX and not in GLP-treated plants. The amide amino acids showed no differences between the genotypes; for both wild-type and mutant plants, IMX induced a similar increase in content whereas levels in GLP-treated plants were indistinct from the controls.

GLP increased Ala content in wild-type plants, but such an increase was much lower in the *adh* mutant

and no significant change was observed in the *aox1a* mutant (Fig. 4).

Total free amino acid content in wild-type plants treated with IMX or GLP was 10 and 6-fold of the content of untreated plants, respectively (Fig. 4). The effects of IMX and GLP lead to different results in mutants. Interestingly, the effect of IMX was less pronounced in mutants (i.e., in mutants it induced a lesser increase compared to untreated plants) and GLP treatment had no effect on mutants as the values remained similar to their control (Fig. 4).

GABA (γ–aminobutyric acid) is a non-protein amino acid that is usually accumulated under stress situations. GABA content showed a non-significant accumulation in GLP treated wild-type plants and this accumulation was not shown in mutant plants (Fig. S3).

Glutathione is a tripeptide (γ-L-glutamyl- L -cysteinlyl-glycine) that is at the heart of the complex antioxidant network (Noctor et al. 2011) and is also important in herbicide detoxification (Dixon and Edwards 2010). We evaluated the possible effect of herbicides on its content. Cys, glu, γ–glutamyl-Cys (first precursor in the synthesis pathway) and total glutathione contents were measured in Arabidopsis roots after 3 days of treatment with IMX or GLP (Fig. 5). Cys content in control plants was higher in *adh* mutant genotype compared to wild-type plants. Both herbicides caused a significant increase in cys, glu and γ–glutamyl-Cys content in wild-type plants but they did not induce any effect in either of the mutants. Both IMX and GLP induced an increase of total glutathione in wild-type plants, while in mutants this increase was only observed after IMX treatment.

4. Discussion

4.1 IMX and GLP cause similar effects on Arabidopsis as on pea

This study investigates the biochemical effects (fermentation, carbohydrate and amino acid content) of two types of herbicides that inhibit the biosynthesis of amino acids in Arabidopsis roots. In contrast to previous studies which focused on the effects these herbicides have on crop species, the use of Arabidopsis allowed us to investigate the impact of specific metabolic pathways on the physiological response of the plants because mutants of this species have been well-characterized.

Induced enzymatic activities of ethanol fermentation was observed in wild-type plants treated with IMX and GLP under aerobic conditions (Fig. 1), as has previously been reported in pea plants treated with ALS-inhibitors (Zabalza et al. 2005) or GLP (Orcaray et al. 2012).

Induced expression of AOX is another common physiological effect described for other species treated with ALS -inhibiting herbicides (Aubert et al. 1997; Gaston et al. 2003) or EPSPS (Zhu et al. 2008). Nevertheless, in our case, only the herbicide IMX produced an induction of the relative expression of AOX genes within the time investigated (Fig. 2).

Moreover, carbohydrates accumulated in roots after the application of amino acid biosynthesis -inhibiting herbicides, as we observed increased fructose, glucose and sucrose contents after GLP treatment and starch accumulated in wild-type plants after IMX application (Fig. 3). Similarly, carbohydrates accumulated in roots of pea plants treated with ALS-inhibitors (Zabalza et al. 2004) and GLP (Orcaray et al. 2012). Carbohydrate accumulations are not only detected in sinks but also in source organs (Zabalza et al. 2004; Orcaray et al. 2012). In these previous studies accumulation in sink and sources was explained as follows: As growth is arrested, carbohydrates are not used in the sinks and they accumulate. The increase in sucrose and starch content in sinks suggests that sucrose is transported from the leaves to the roots at a higher rate than it is used in the sinks. Under these conditions, the sugar gradient required for long-distance transport is abolished; thus, phloem transport is inhibited. As a consequence, carbohydrate accumulation in the leaves of treated plants occurred because of a decrease in sink strength (Zabalza et al. 2004).

Although the content of branched-chain and aromatic amino acids with respect to total amino acids was not similar to that previously described in pea plants (ChingYuh 2001; Orcaray et al. 2010, 2012), analysis of amide and acidic amino acids did produce similar data for the two plant species. Furthermore, an accumulation of total free amino acids was detected in plants treated with either of the herbicides (Fig. 4). This physiological effect had previously been described after application of ALS-inhibitors (Anderson and Hibberd 1985; Shaner and Reider 1986; Rhodes et al. 1987; Scarponi et al. 1995; Zabalza et al. 2005, 2011; Zhou et al. 2007; Orcaray et al. 2010) and GLP treatments (ChingYuh 2001; Orcaray et al. 2010, 2012). (Rhodes et al. 1987) proposed that the observed accumulation of free amino acids in treated plants is due to an increased protein turnover. Evaluation of the effects of IMX and GLP on the main proteolytic systems in pea showed that although they were affected by both herbicides, not all of them increased (Zulet et al. 2013a).

Glutathione is a simple sulfur compound composed of three amino acids; its functions are manifold, but notably include redox-homeostatic buffering (Noctor et al. 2011). Roots of wild-type plants treated with IMX or GLP showed an increase in total glutathione content (Fig. 5), as has been reported previously in pea plants treated with another ALS-inhibitor (Zabalza et al. 2008). The increase in homoGSH detected in soybean treated with GLP was related to a transient oxidation (Vivancos et al. 2011).

Collectively these results indicate that *Arabidopsis thaliana* shows typical physiological markers as toxic consequences of amino acid biosynthesis -inhibiting herbicides which have been reported previously in other species with some deviations. The confirmed effects on Arabidopsis wild-type plants support the use of mutants to evaluate the physiological effects of these herbicides in mutant genotypes that lack fermentation or alternative oxidase. These physiological effects are used in the present work as markers of herbicide effect, and therefore toxicity has been compared through these markers.

4.2 Characterization of adh and aox mutants under control conditions

To investigate the impact of fermentation and alternative respiration on herbicidal activity, T-DNA knock-out lines for ADH1 and AOX1a were characterized. First, untreated control plants of each genotype used in this work were compared in order to ascertain whether or not any differences were due to the treatment or to the lack of fermentation / AOX pathways. No phenotypical differences were found between the different lines used. Although at the onset of the treatment photosynthesis rate was lower in *aox1a* plants than in the two other genotypes, during the time of study controls for wild-type and both mutant plants presented fluctuation around the same values for photosynthesis indicators (Supplemental Fig. 1). This also happened for almost all the other parameters measured, except PDC activity, cysteine and carbohydrate content (Figs. 1, 3 and 5). In general, a lack of *AOX1a* does not affect the growth or phenotype (Fiorani et al. 2005; Watanabe et al. 2008; Strodtkötter et al. 2009; Yoshida et al. 2011; Gandin et al. 2012), but it does affect the stress response (Giraud et al. 2008; Yoshida and Noguchi 2011). Similarly, *adh* null mutants (Dolferus et al. 1997) have demonstrated less tolerance (Ellis et al. 1999) to stress induced by hypoxia that wild-type plants.

4.3 Physiological effects in mutant plants

In this study, expression of two of the four genes of the PDC gene family (*PDC1* and *PDC2*) in *Arabidopsis thaliana* was determined (Fig. 1). Contrary to previously published evidence (Kursteiner et al. 2003), it has recently been shown that not only *PDC1* but also *PDC2* plays a role in the ethanol fermentation pathway under hypoxic conditions (Mithran et al. 2014). Our results show a different pattern for both genes following herbicide treatment in mutant plants. Induction of *PDC2* transcription after herbicide treatment was detected in mutant but not in wild-type plants. Contrastingly, the induction of *PDC1* relative expression level detected in wild-type plants after IMX treatment was not observed in either of the mutants. As *PDC1* is predominantly present in roots, while *PDC2* has been shown to be leaf-specific (Mithran et al. 2014), PDC activity and amount of protein should be correlated with the *PDC1* expression pattern. Nevertheless, both mutant and wild-type plants showed induced PDC activity and protein levels after IMX treatment. So,

although IMX had no effect on PDC1 expression in the mutants, enzymatic activity was induced by IMX; suggesting that an unknown post-transcriptional regulatory mechanism is counteracting the lack of effect on the transcription.

Ethanol fermentation has been suggested to play an important role in aerobic metabolism under stress conditions (Tadege et al. 1999), supporting the conclusion that this pathway could act as an overflow pathway in certain situations. Although under aerobic conditions this role has recently been related to PDC and not to ADH (Mithran et al. 2014), our results show that IMX produced a parallel induction of *PDC1* and *ADH1* expression in Col-0 wild-type and *aox1a* genotypes.

Other species treated with ALS-inhibitors have also demonstrated an induction of AlaAT activity (Zabalza et al. 2005), but not after the application of GLP (data not shown). Similarly, the relative expression level of *AlaAT2* in wild-type plants increased significantly in those treated with IMX but not after GLP treatment (Fig. 1). This can be explained by the fact that both AlaAT and ALS enzymes share the same substrate, pyruvate. The inhibition of ALS activity by IMX would increase pyruvate availability for other enzymes, whereas GLP does not have such a direct effect on pyruvate availability. The increase in *AlaAT2* detected in wild-type plants treated with IMX was also observed in both mutants, proving that this physiological effect of ALS-inhibitors is not modified if fermentation or AOX is inhibited.

In wild-type plants an increase in the relative expression levels of the hypoxia marker *HB1* gene was induced after treatment with either of the herbicides (Fig. 1). However, this increase was not observed in both mutants, thus indicating that the common trigger to induce expression of these hypoxia responsive genes in the wild-type plants is no longer present in the mutants treated with the herbicides.

This study shows that the induction of increased *PDC1* and *HB1* expression detected in wild-type species treated with herbicides was reduced or less severe in the mutants (Fig. 1), therefore, it can generally be stated that the induction of aerobic ethanol fermentation detected after amino acid biosynthesis inhibition is alleviated in plants lacking *ADH1* or *AOX1a.*

As can be seen in Figure 2, we confirmed that *aox1a* did not express transcripts for this gene. Furthermore, we confirmed that the difference in the transcript levels of other AOX isoforms was small between the wild-type and *aox1a* plants, suggesting that there was no complementation of the *AOX1a* deficiency by other AOX isoforms (Watanabe et al. 2010). Moreover, *AOX1a* gene expression was the most abundant in roots (Fig. 2), as has been reported before (Clifton et al. 2006). Although the physiological role of this pathway remains to be clarified, several clues on the function of AOX have been revealed in recent years. AOX can help to alleviate the effects of several stresses, avoiding the over-reduction of the electron transfer chain while reducing the electron flow through the cytochrome pathway (Vanlerberghe 2013). In this sense, AOX induction has been related to stresses in which the cytochrome pathway is restricted (Vanlerberghe and McIntosh 1997; Parsons et al. 1999; Amor et al. 2000; Vanlerberghe et al. 2002).

Our results indicate that plants treated with aromatic or branched-chain amino acid biosynthesis - inhibiting herbicides induced less efficient ATP-producing pathways, alternative respiration and ethanol fermentation. Moreover, as said before, as pyruvate can be related with the induction of fermentation and can

also act as an allosteric activator of AOX; it can be proposed that fermentation and AOX could play a role in the faster metabolization of a greater pyruvate availability.

The carbohydrate accumulation detected in treated wild-type plants (Fig. 3) confirms the accumulation observed after the application of ALS-inhibitors and GLP (Zabalza et al. 2004; Orcaray et al. 2012). However, the carbohydrate accumulation we detected in wild-type plants was less pronounced in the mutants and, in the case of total soluble carbohydrates, there was even less accumulation in mutants after the application of GLP in comparison with untreated plants. This leads to the conclusion that plants lacking *ADH1* and *AOX1a* do not show this herbicide marker.

Total free amino acid content showed a similar pattern for both herbicides in the wild-type species, but differences were observed between GLP and IMX when they were applied to the mutants (Fig. 4). In wild-type plants, an increase in the total free amino acid content was observed after the application of both IMX and GLP; in the mutants, an increase was only observed with IMX (however, increases were less prominent compared to the wild-type) and GLP application prompted no change as the total free amino acid content was similar to untreated control plants. Our results showed that free amino acid accumulation was less noticeable or no detected in both *adh* and *aox1a* mutants when treated with IMX or GLP herbicides, respectively; thus indicating that plants which lack fermentation or AOX diminish the effects of these herbicides on this physiological effect.

Herbicide efficacy has been associated with carbon-nitrogen imbalances after comparing the physiological effects of inhibitors of two consecutive enzymes in the biosynthesis of branched-chain amino acids: ALS and ketol-acid reductoisomerase (KARI, EC 1.1.1.86) (Zabalza et al. 2013). Only ALS inhibitors have shown enough herbicidal efficacy to be commercialized. Only the ALS inhibitor induced an increase in the free amino acid content, indicating that the imbalance in the carbon/nitrogen metabolism induced after ALS inhibition is one of the reasons of higher phytotoxicity (Zabalza et al. 2013). In this context, free amino acid accumulation induced by herbicides in wild-type plants were alleviated in the mutants, indicating that plants which lack *ADH1* or *AOX1a* s less susceptibility to the herbicides' phytotoxicity.

Expression of glutathione and its precursors (cys, glu and γ−glutamyl-Cys) showed similar patterns in the wild-type plants: the content of each of them increased after IMX and GLP application (Fig. 5). However, these increases were not observed in any of the cases of treated mutant plants, except for the glutathione content which produced similar results after IMX in all genotypes. However, these results show that most of the physiological effects induced by herbicides in wild-type plants were alleviated in the mutants, thus confirming again that plants which lack *ADH1* or *AOX1a* show less susceptibility to the herbicides' action.

Treated wild-type Arabidopsis plants showed the typical physiological markers produced in response to ALS or EPSPS -inhibiting herbicides: aerobic fermentation and alternative oxidase induction; soluble carbohydrate and total free amino acid accumulation. Induction of fermentation and alternative oxidase were more prominent after IMX treatment compared to GLP, while GLP induced a greater increase in carbohydrate content. Both herbicides caused increased free amino acid content. A lack of *ADH1* or *AOX1a* genes seems to change plant response after stress application in the form of these herbicides, as the common physiological

effects described for amino acid biosynthesis -inhibiting herbicides are less prominent or abolished in mutant plants. In general, the induced effects after herbicide treatment detected in fermentation, carbohydrate and amino acid content and an alternative oxidase pathway in wild-type plants were alleviated (or in some cases not exacerbated) in mutant plants, noting that the level of relief detected was more evident for GLP than for IMX.

Fermentation and AOX have been described as alternative metabolic pathways for excess pyruvate; therefore their induction in treated plants would result in better tolerance to the herbicide by facilitating greater control over pyruvate content. Contrarily, our results indicate that the induction of fermentative and alternative oxidase pathways is a consequence of the toxicity of the herbicides, because the physiological imbalances are not as pronounced in mutant lines.

The results of this study show profound impact on the content of major metabolites after herbicide treatments in wild-type roots and in many cases this impact was not as profound in mutants. Nevertheless, as lethality was similar in the three genotypes the precise role of fermentation and alternative oxidase in the toxic effects of GLP and IMX can not be outlined.

The repeated use of herbicides with the same mechanism of action is increasing the selection pressure with the consequence of producing ever more resistant weeds in recent years. Therefore novel herbicides with new biochemical target sites are needed to alleviate the selection pressure by attacking a different target in the plant's metabolism (Duke 2012). Investigation into the processes that lead to lethality of current herbicides can provide useful information about the metabolic points involved in the plant's response to the herbicidal activity, thus determining whether the mechanisms increase or decrease the herbicide toxicity. In this context this study provides new insights in the processes following application of two popular herbicides with different targets.

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Figure legends

Fig. 1. Fermentation. A) Relative expression levels (transcripts) of the genes *PDC1, PDC2, ADH1, LDH1, AlaAT2* and *HB1* in roots of *Arabidopsis thaliana* plants (wild-type Col-0 and mutants *adh* and *aox1a*) untreated (control) and treated with imazamox or glyphosate (3 days after application). Each value is the mean ± standard error (n = 3). For each genotype, ● and ● indicate significant differences between control and imazamox or glyphosate treated plants, respectively (*p* value ≤0.05).

B) PDC and ADH immunoblots and activities of *Arabidopsis thaliana* root genotypes Col-0, *adh* and *aox1a*, control and treated with imazamox or glyphosate at day 3 after treatment. Each value is the mean ± standard error (n = 3 - 6). The symbol ▽ highlights significant differences between control plants (without herbicide) from mutant lines with respect to wild-type control plants (p≤0.05). For each genotype, ● and ● indicate significant differences between control plants and imazamox or glyphosate treated plants, respectively (*p* value ≤0.05).^ indicates significant differences (p≤0.1) between control and treatment. For the Western blots, 15 µg of protein were loaded into each well.

Fig. 2. Alternative oxidase. Relative expression levels (transcripts) of the genes *AOX1a, AOX1b, AOX1c, AOX1d* and *AOX2* in roots of *Arabidopsis thaliana* plants (wild-type Col-0 and mutants *adh* and *aox1a*) untreated (control) and treated with imazamox or glyphosate (3 days after application). For each genotype, ● and ● indicate significant difference between control and imazamox or glyphosate treated plants, respectively (*p* value ≤0.05).

Fig. 3. Carbohydrates. Fructose, glucose, sucrose and starch contents in roots of *Arabidopsis thaliana* plants (wild-type Col-0 and mutants *adh* and *aox1a*) untreated (control) and treated with imazamox or glyphosate (3 days after application). Each value is the mean ± standard error (n = 3). The symbol ▽ highlights significant differences between control plants (without herbicide) from mutant lines with respect to wild-type control plants (p≤0.05). For each genotype, ● and ● indicate significant differences between control and imazamox or glyphosate treated plants, respectively (*p* value ≤0.05).

Fig. 4. Free amino acids. Branched-chain, aromatic, acidic and amide amino acid content with respect to the total free amino acids (percent of the total free amino acid), alanine content (ALA) and total free amino acid content in roots of *Arabidopsis thaliana* plants (wild-type Col-0 and mutants *adh* and *aox1a*) untreated (control) and treated with imazamox or glyphosate (3 days after application). Each value is the mean ± standard error (n = 3). For each genotype, ● and ● indicate significant differences between control and imazamox or glyphosate treated plants, respectively (*p* value ≤0.05).

Fig. 5. Glutathione and its precursors. Cysteine, glutamic acid, γ–glutamyl-Cys and total glutathione contents in roots of *Arabidopsis thaliana* plants (wild-type Col-0 and mutants *adh* and *aox1a*) untreated (control) and treated with imazamox or glyphosate (3 days after application). Each value is the mean ± standard error (n = 3). The symbol ▽ highlights significant differences between control plants (without herbicide) from mutant lines respect to wild type (*p* value ≤0.05). For each genotype, ● and ● indicate significant differences between control and imazamox or glyphosate treated plants, respectively (*p* value ≤0.05).

Figure 1

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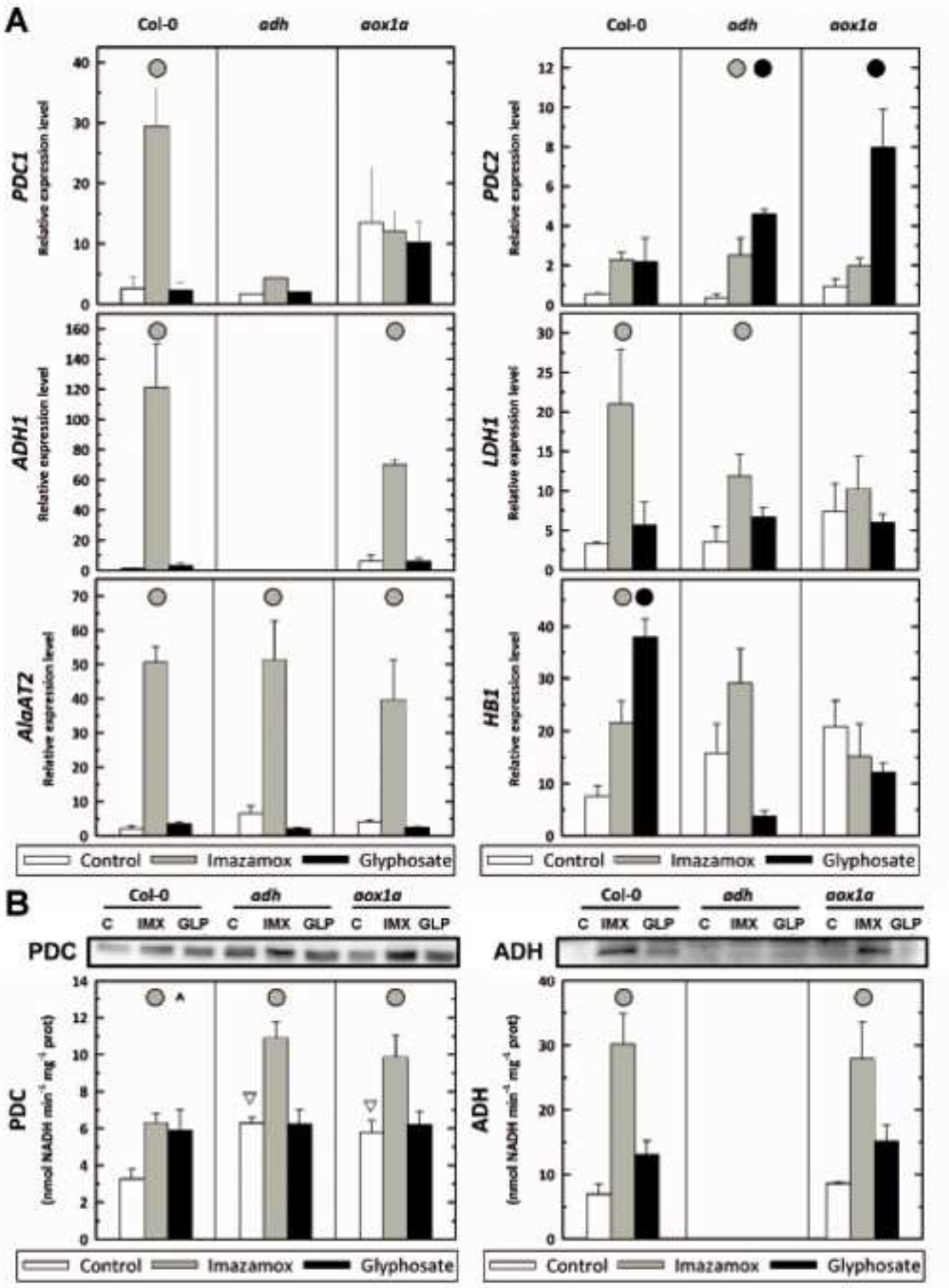

Figure 1
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Figure 2

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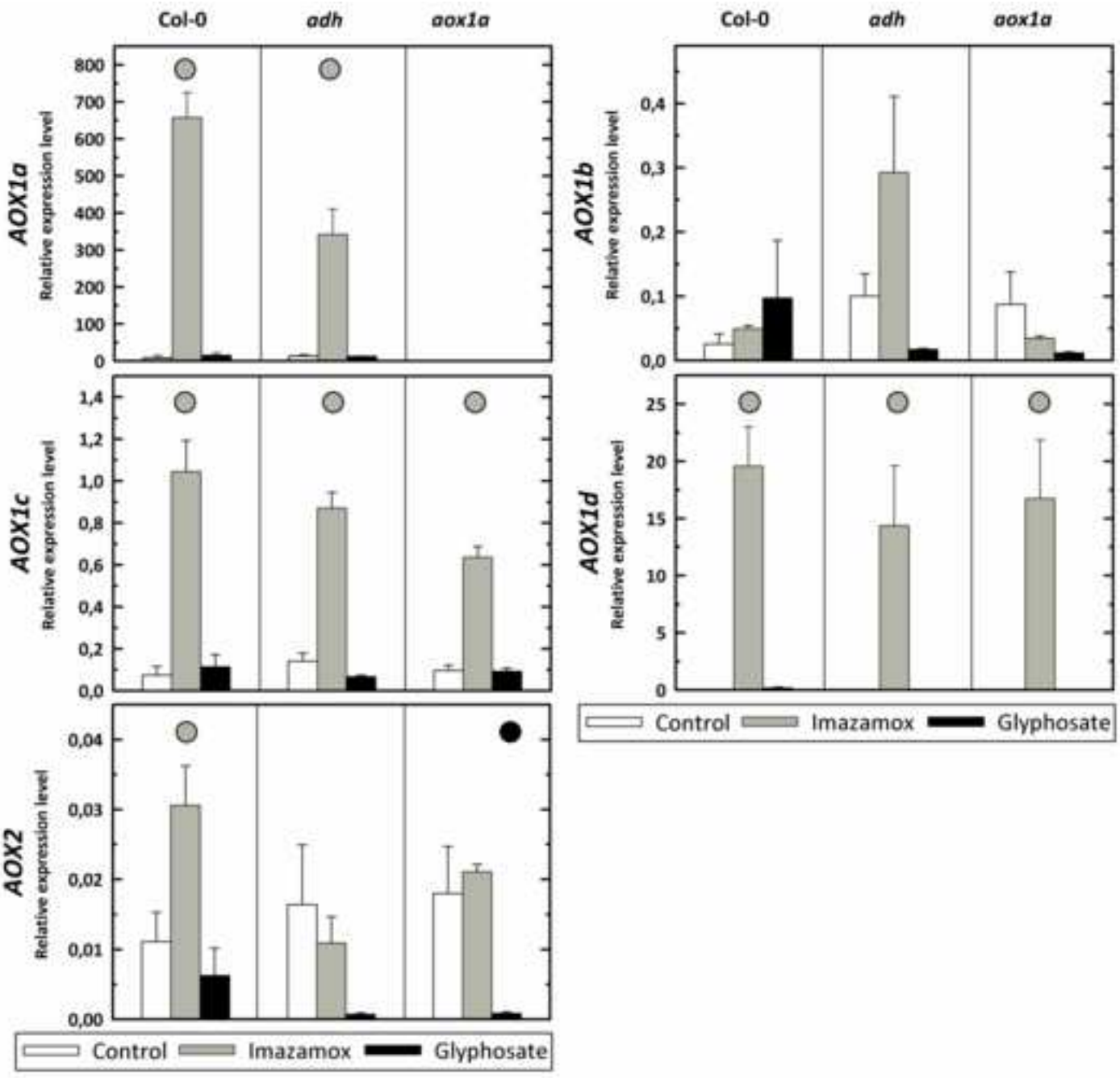

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Figure 3

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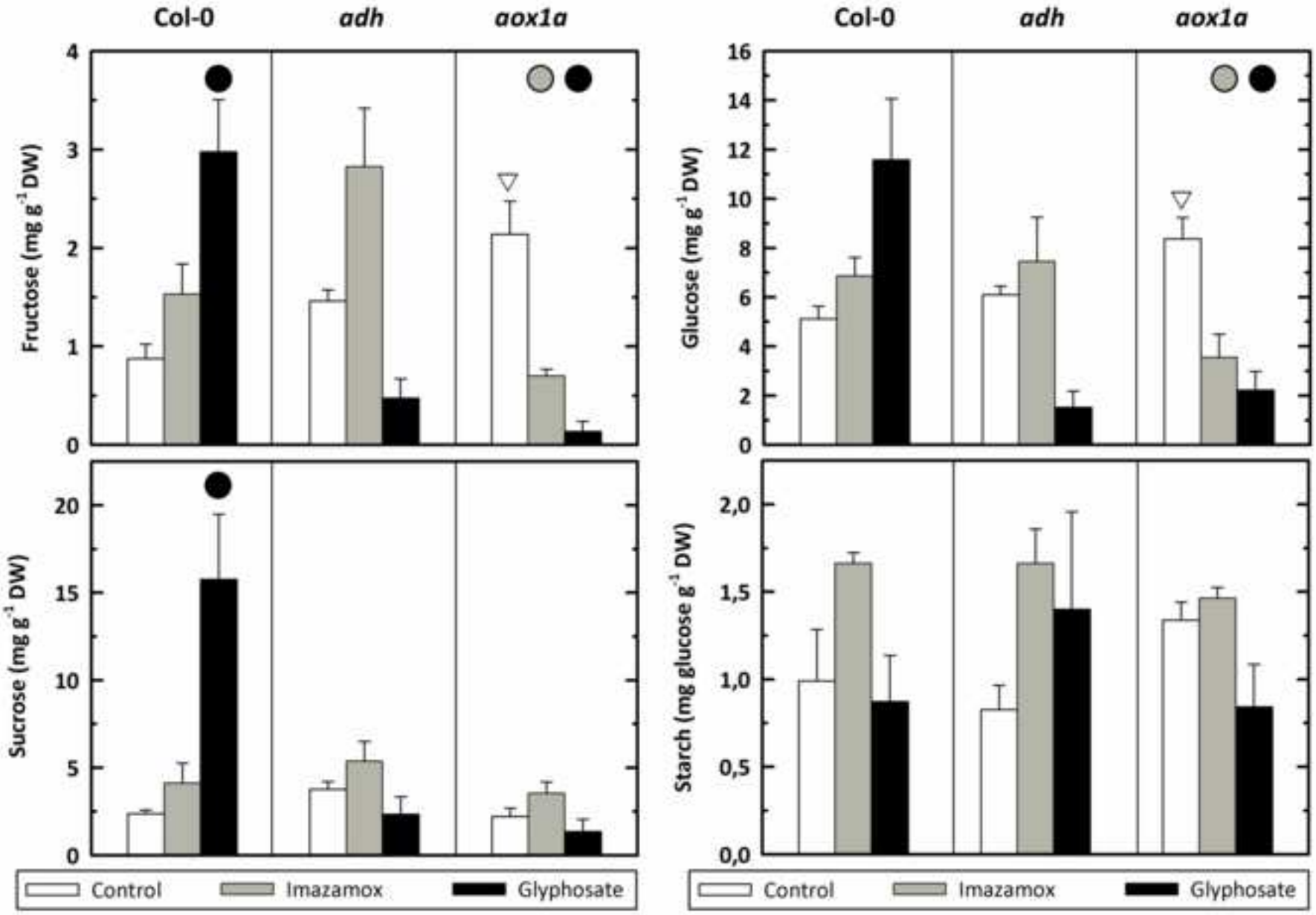

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Figure 4

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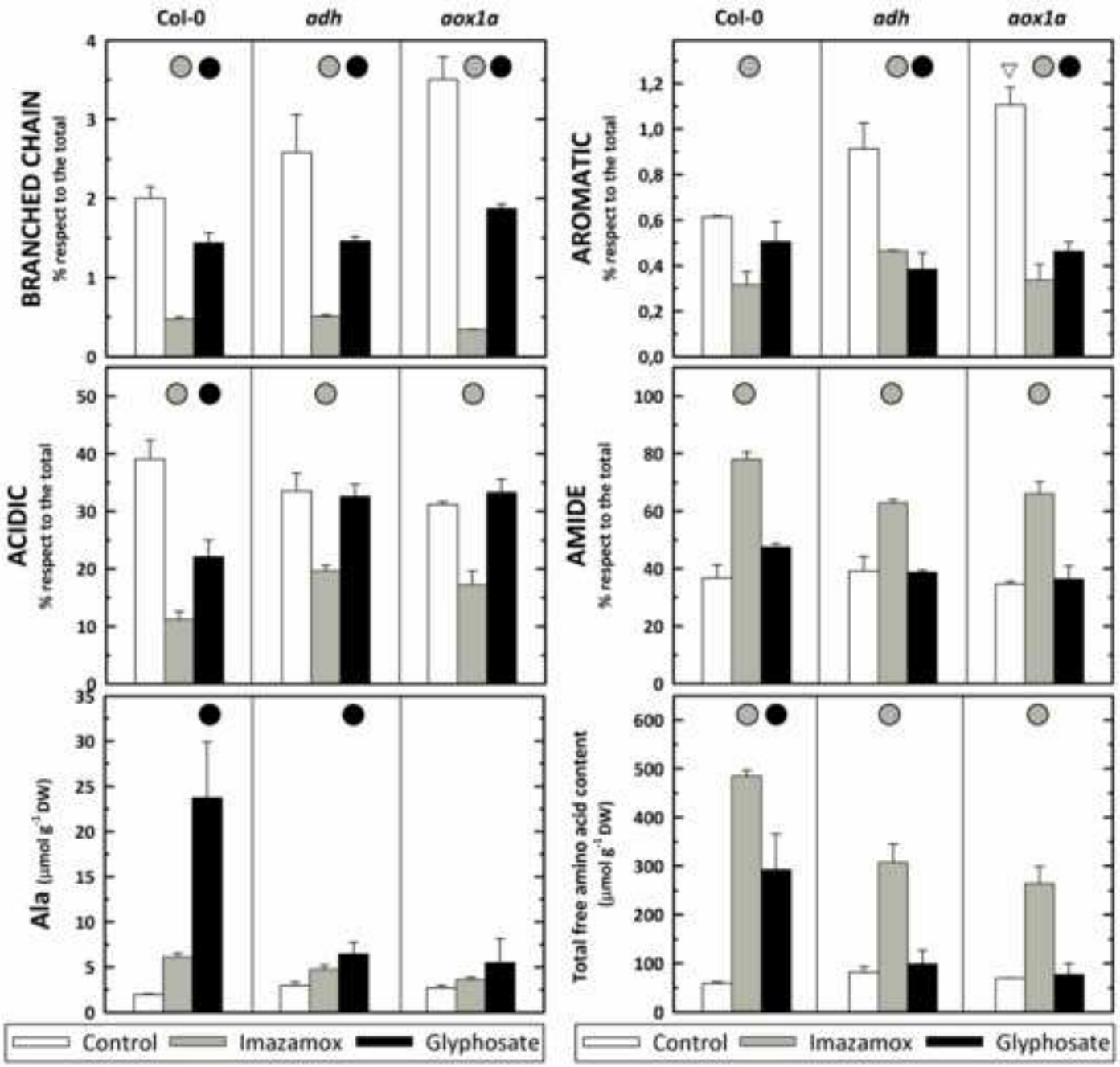

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Figure 5

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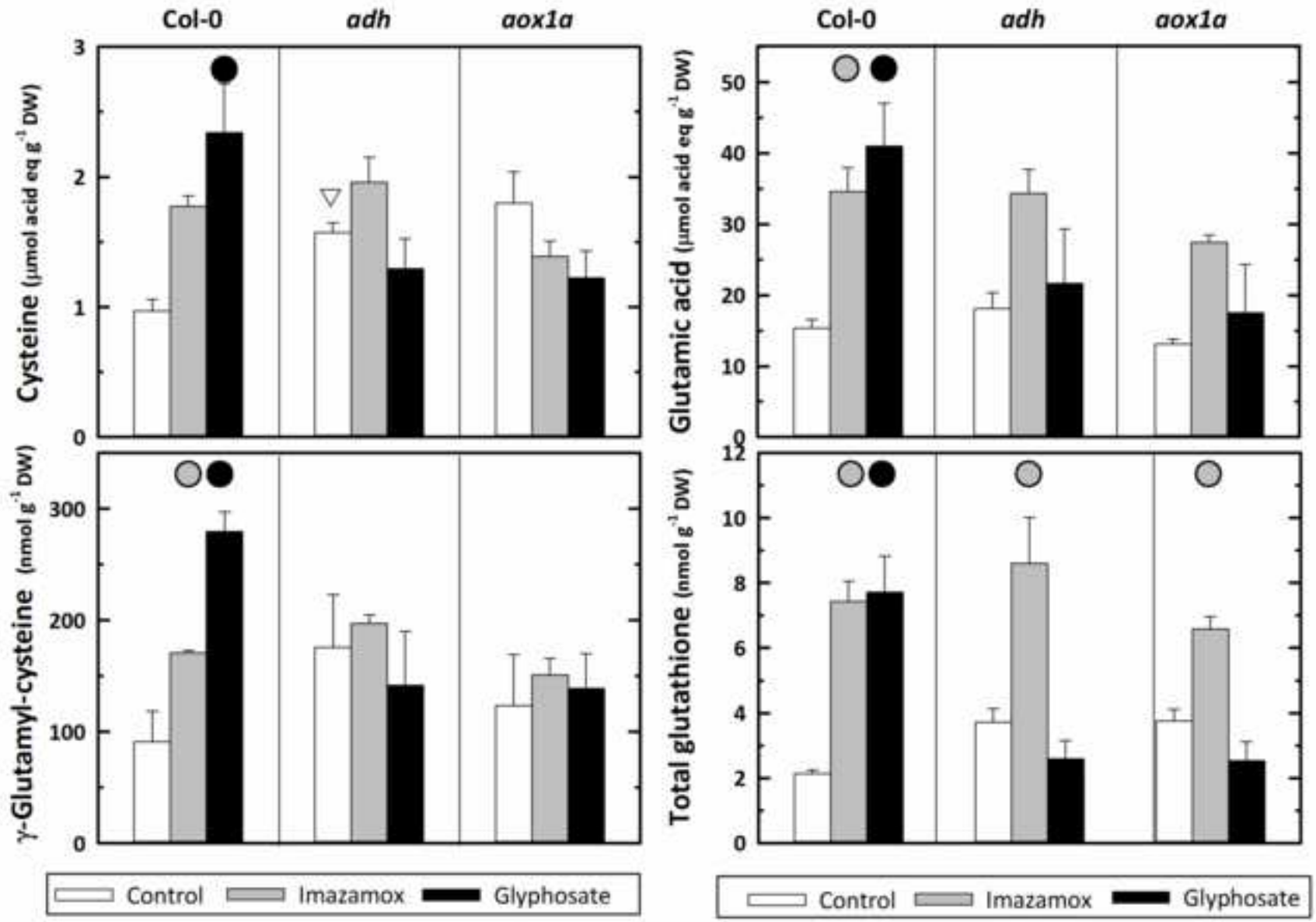

Figure 5
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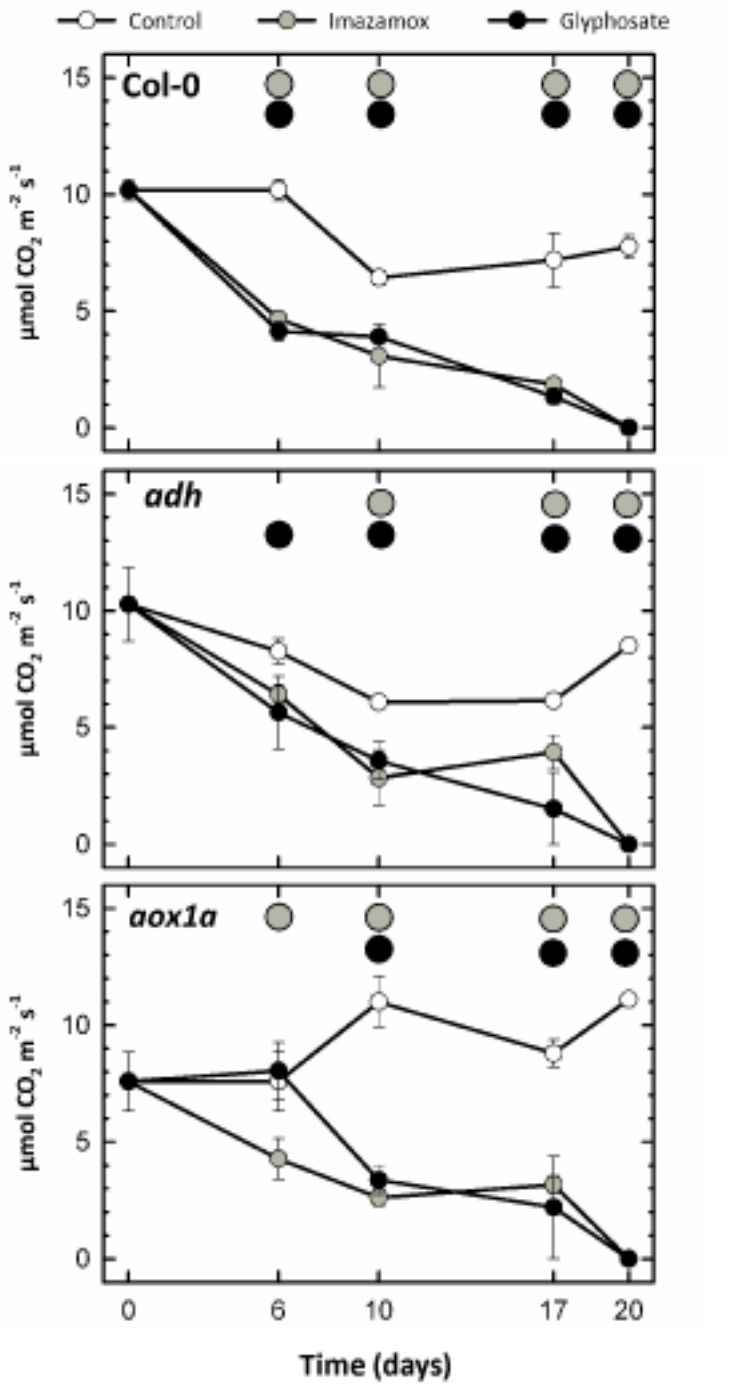
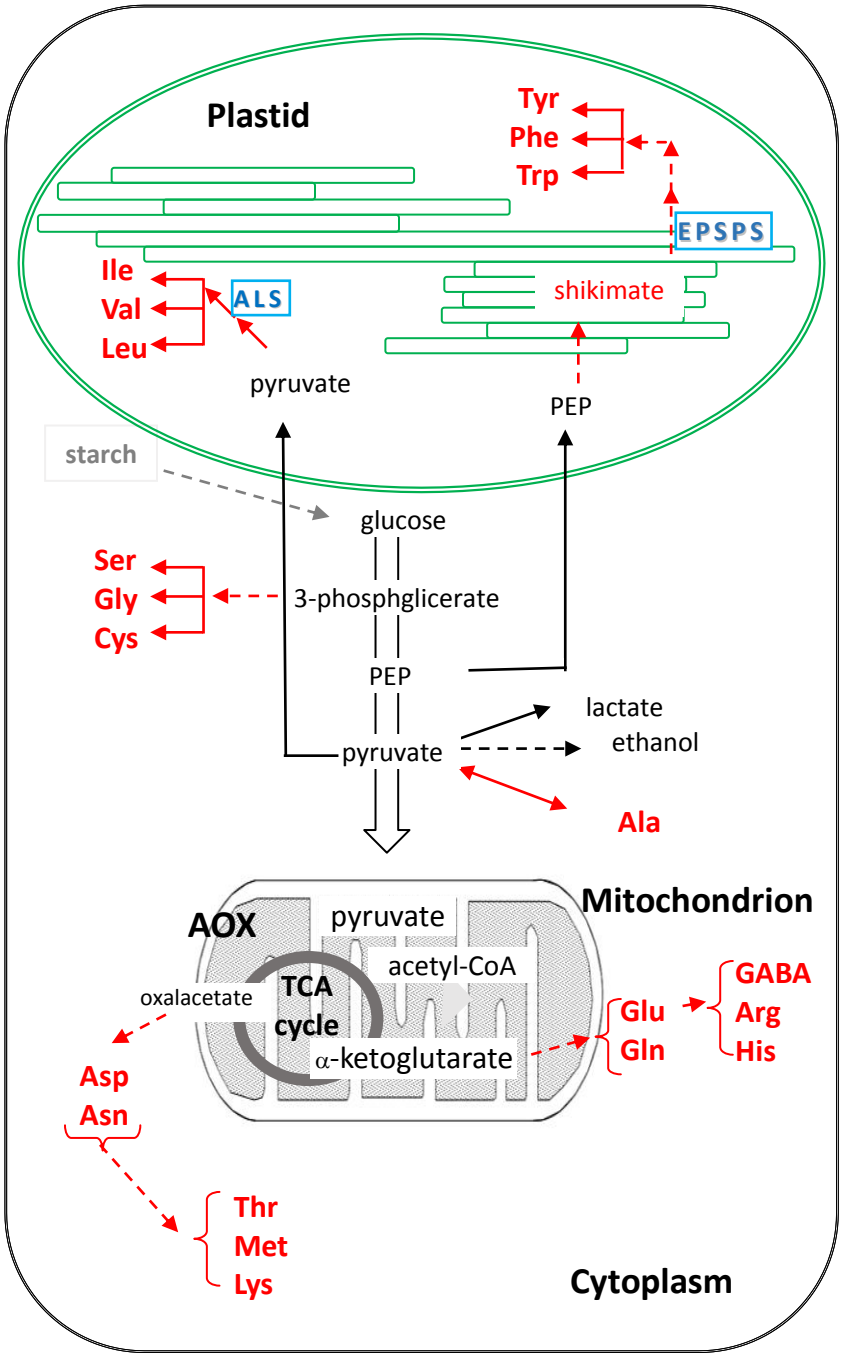
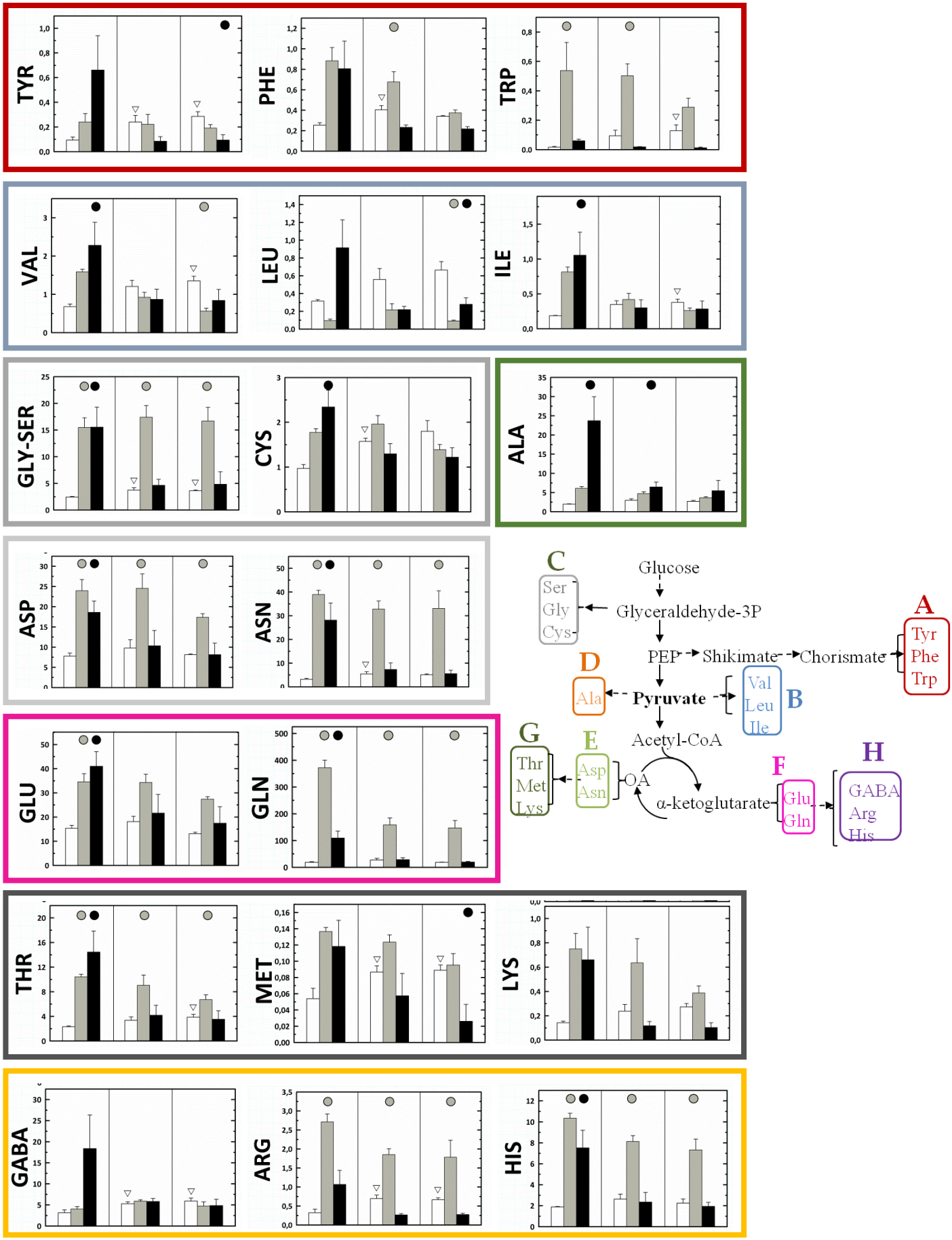
Supplemental Figure 1. Net photosynthesis in leaves of untreated *Arabidopsis* (wild-type Col-0 and mutants *adh*- and *aox1a*) plants or plants treated with imazamox or glyphosate applied to the nutrient solution. Measurements were made on the youngest, fully-expanded leaf. Each value is the mean ± standard error (n = 10 - 15). ● and ● indicate significant differences between control and imazamox or glyphosate treated plants, respectively (p value≤0.05), for a given day.



Supplemental Figure 2. Overview of the cellular localization of the target sites of herbicides inhibiting amino acid biosynthesis (shown in blue) and simplified view of the amino acid biosynthesis. Pyruvate plays a central role in linking fermentative metabolism and amino acid biosynthesis. ALS acetolactate synthase, EPSPS 5-enolpyruvyl-shikimate-3-phosphate synthase, AOX alternative oxidase, PEP Phosphoenol pyruvate.





Supplemental Figure 3. Amino acid content in roots of *Arabidopsis thaliana* plants (wild-type Col-0 and mutants *adh*- and *aox1a ko*) untreated (control) and treated with imazamox or glyphosate (3 days after application). Mean ± SE (n = 3). The symbol▽ highlights significant differences between control plants (without herbicide) from mutant lines with respect to wild-type control plants ($p \leq 0.05$). For each genotype, ● and ● indicate significant differences between control and imazamox or glyphosate treated plants, respectively ($p \leq 0.05$). OA:oxaloacetate.